

**IN THE SPECIFICATION:**

Please replace the paragraphs beginning at page 3, line 19, with the following amended paragraphs:

According to a first aspect of the invention, there is provided a yeast cell containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to a heterologous ~~inducible~~ inducible promoter.

We have found that yeast cells containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to an ~~inducible~~ inducible promoter (e.g. the cells according to the first aspect of the invention) may be used in applications where the induction of cell lysis is desirable. For instance, induction of yeast cell lysis is useful for isolating protein expressed within a yeast cell which is not readily secreted into the medium in which the cells are growing. Thus according to a second aspect of the present invention, there is provided a method of regulating yeast cell lysis comprising:

(i) growing yeast cells containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to an ~~inducible~~ inducible promoter in a growth medium which activates the ~~inducible~~ inducible promoter such that *SRB1/PSA1* and *PKC1* are expressed from said cells; and

(ii) when lysis is required, growing the cells in a modified growth medium which represses *SRB1/PSA1* and *PKC1* expression such that cell lysis is induced.

The present invention is based upon our efforts to develop conditional lysis mutants that do not require special media in which to grow (e.g. sorbitol supplemented) or temperature shifts.

We placed various genes that have been shown to contribute, in different ways, to cellular integrity of *S. cerevisiae* under the control of ~~inducible~~ inducible promoters and examined whether or not repression of the gene (by altering the composition of the media in which the yeast is grown such that the promoter is inactivated) modulates cell lysis. We found that repression of some of the genes tested, for instance *PDE2*, did not significantly influence lysis. These genes were therefore unsuitable candidates to be modulated to generate an improved lytic yeast strain.

Please replace the paragraphs beginning at page 5, line 16, with the following amended paragraphs:

Cells according to the first aspect of the invention may be formed from yeast strains with normal *SRB1/PSA1* and *PKC1* expression. Preferably the yeast is *Saccharomyces cerevisiae* or strains thereof. Examples of such yeast strains include ZO123 and FY23. The conservation of gene function in different yeast species means that other types of yeasts (particularly those that are currently exploited for heterologous gene expression such as *Pichia pastoris*, *Hansenula polymorpha* and *Kluyveromyces lactis*) may be used to form cells according to the first aspect of the invention in which their *SRB1/PSA1* and *PKC1* homologues are operatively linked to an ~~inducible~~ inducible promoter.

The endogenous promoters of the *SRB1/PSA1* gene and the *PKC1* gene are not readily ~~inducible~~ inducible in such strains and it is therefore necessary to modify genetically yeast such that *SRB1/PSA1* and *PKC1* expression from the yeasts is ~~inducible~~ inducible. This may be

achieved in a number of ways. For instance, yeast cells may be transformed with DNA molecule(s) comprising an ~~inducible~~ inducible promoter(s) such that the ~~inducible~~ inducible promoter(s) take over control of transcription of the endogenous *SRB1/PSA1* and *PKC1* genes. These DNA molecules are preferably designed such that they will integrate into the yeast genome and replace the region of DNA containing the endogenous *SRB1/PSA1* and *PKC1* promoters (as appropriate). As a result the ~~inducible~~ inducible promoter introduced into the cell becomes operatively linked to the *SRB1/PSA1* and *PKC1* genes and can control their expression. Alternatively the cells may be transformed with a first recombinant DNA molecule comprising an ~~inducible~~ inducible promoter operatively linked to the *SRB1/PSA1* gene and/or a second recombinant DNA molecule comprising an ~~inducible~~ inducible promoter operatively linked to the *PKC1* gene. These recombinant DNA molecules are designed such that they will integrate and replace by homologous recombination the endogenous *SRB1/PSA1* and *PKC1* genes respectively. The DNA molecules and recombinant DNA molecules used for transforming yeast cells are preferably incorporated in a suitable vector which bears a DNA sequence which allows homologous recombination between the vector and the DNA at the site of the endogenous promoter / gene.

The cells according to the first aspect of the invention may also be derived from yeasts which are *srb1-1* and/or *pkc* mutants. These mutants have a lytic phenotype and are only able to survive when grown in osmotically buffered media. However we have found that these cells may be transformed with an expression cassette comprising an ~~inducible~~ inducible promoter and DNA sequences encoding suitable genes to replace the mutated gene to form cells according to the first aspect of the invention which display a normal phenotype (i.e. they are not osmotically

sensitive or liable to lyse spontaneously) in permissive growth media conditions (which allows activation of the ~~inducible~~ inducible promoter) but will lyse when the media is modified such that gene expression is repressed. Yeast cells to be modified may be transformed with the abovementioned recombinant DNA molecules (or vectors bearing such molecules) to form cells according to the first aspect of the invention in which the recombinant DNA molecules either integrate into the genome of the mutant yeast or which may subsist (and ideally autonomously replicate) in the cytosol of the yeast cell. Examples of *srb1-1* and/or *pkc* mutant cells which may be used include the ZO124 strain of *Saccharomyces cerevisiae*.

The *SRB1/PSA1* gene and the *PKC1* gene (or functional derivatives thereof) may each be operatively linked to a number of ~~inducible~~ inducible promoters. The ~~inducible~~ inducible promoter may, for example, be the *GAL1* promoter (~~inducible~~ inducible by galactose) or the *TET* promoter (~~inducible~~ inducible by tetracyclin).

Please replace the paragraphs beginning at page 9, line 14, with the following amended paragraphs:

We have found that lysis may be regulated according to the method of the second aspect of the invention by growing yeast cells according to the first aspect of the invention in a growth medium which activates the ~~inducible~~ inducible promoter such that *SRB1/PSA1* and *PKC1* are expressed from said cells. Then, after a predetermined time, the cells may be switched to growth in the modified growth medium such that *SRB1/PSA1* and *PKC1* expression is repressed and cell lysis induced.

The manner in which *SRB1/PSA1* and *PKC1* gene expression is regulated according to the method of the second aspect of the invention will depend upon which ~~inducible~~ inducible promoter is being used. This regulation is dependent upon the exact concentration of an agent capable of modulating promoter activity contained within the growth medium. We have found that addition to the media of methionine to a concentration of between 0.05mM and 20mM will inhibit expression of *SRB1/PSA1* and *PKC1* from cells transformed with *pMET3-SRB1* and *pMET3-PKC* and thereby induce lysis whereas the same cells grown in the absence (or minimal concentration) of methionine are able to grow unimpeded. Preferably a concentration of between about 0.05mM and 5mM methionine in the media and most preferably a concentration of about 2mM methionine in the media is used to induce lysis.

The growth medium used according to the method of the second aspect of the invention should be readily adaptable such that it may be in either of two forms: one which permits activation of the ~~inducible~~ inducible promoter and thereby *SRB1/PSA1* and *PKC1* expression; and a second form which is modified such that *SRB1/PSA1* and *PKC1* gene expression is repressed. This repression may be effected by removal of an agent which activates the promoter but is preferably effected by addition to the media of an agent which inhibits the promoter.

The growth medium should contain sufficient amounts of nutrients (i.e carbohydrate, nitrogen source etc) required to allow optimal growth of yeast when *SRB1/PSA1* and *PKC1* are not being repressed.

The exact composition of the medium depends upon a number of factors (for instance the specific yeast used). Purely by way of example a suitable growth medium is F1 medium which comprises:

<u>Mineral salts</u>	final concentration in F1-medium
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Ammonium sulphate	3.13 g/l
Potassium dihydrogen orthophosphate	2.00 g/l
Magnesium sulphate 7-hydrate	0.55g/l
Sodium chloride	0.10g/l
Calcium chloride dihydrate	0.09g/l

<u>Trace elements</u>
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Zinc sulphate 7-hydrate	0.07mg/l
Ferric chloride 6-hydrate	0.05mg/l
Cupric sulphate	0.01mg/l
Boric acid	0.01mg/l
Potassium iodide	0.01mg/l

<u>Vitamins</u>
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Inositol	62.00mg/l
Thiamine Hydrochloride	14.00mg/l
Pyridoxine	4.00mg/l
Calcium Pantothenate	4.00mg/l
d-Biotin	0.30mg/l

+ carbohydrate substrate

+/- agent which modulates the ~~inducable~~ inducible promoter

The composition of the media and the modified form thereof ideally only differ by the inclusion or exclusion of an agent which modulates the ~~inducable~~ inducible promoter. The type of agent used will depend upon which specific promoter is used. When cells are used in which *SRB1/PSA1* and *PKC1* are operatively linked to the *pMET* promoter, the media permissive for yeast cell growth should be free of methionine. Methionine may be added to the medium as required to form the modified media in which lysis is induced.

Please replace the paragraphs beginning at page 12, line 26, with the following amended paragraphs:

According to a third aspect of the present invention, there is provided a method of regulating yeast cell flocculation comprising:

- (i) growing yeast cells containing the *PKC1* gene or functional derivatives thereof operatively linked to an ~~inducable~~ inducible promoter in a growth medium which activates the ~~inducable~~ inducible promoter such that *PKC1* is expressed; and
- (ii) when flocculation is required, growing the cells in a modified growth medium which represses *PKC1* expression such that flocculation is induced.

According to the third aspect of the invention we have found that the repression of *PKC1* expression makes it possible to induce flocculation. Therefore cells containing the *PKC1* gene or

functional derivatives thereof operatively linked to a heterologous ~~inducible~~ inducible promoter (such as *pMET3*) are useful when it is desired to induce flocculation. Such cells are particularly useful when the simultaneous induction of lysis and flocculation is required (as *PKC1* repression also causes lysis). The induction of lysis and flocculation is desirable when purifying proteins released from yeasts. The induced lysis liberates the contents of the cell whereas the induced flocculation will favour sedimentation of the cell ghosts / debris and thereby separate cell contents (which will remain in the media) from the cell ghosts / debris.

Cells suitable for use according to the method of the third aspect of the invention include cells in which only *PKC1* is under the regulation of an ~~inducible~~ inducible promoter and include:

- (i) ZO124 transformed with *pRS316-pMET3-PKC1*, *pRS316-F<sub>1</sub>F<sub>2</sub>-pMET3-PKC1* or *pRS316-F<sub>1</sub>F<sub>2</sub>-TRP1-pMET3-PKC1* (see Example 1);
- (ii) ZO123 transformed with *pRS316-pMET3-PKC1* or *pMET3-PKC1* containing fragments derived from *pRS316-F<sub>1</sub>F<sub>2</sub>-pMET3-PKC1* or *pRS316-F<sub>1</sub>F<sub>2</sub>-TRP1-pMET3-PKC1* (see Example 1); and
- (iii) yeast strain ZO-126 (see Example 2).

We have also found that cells may be developed which have a flocculating phenotype by removing *SRB1/PSA1* from under the control of its endogenous promoter and placing the gene under the control of a heterologous promoter (which may be ~~inducible~~ inducible or constitutive). Such cells flocculate but do not lyse to a significant extent and are therefore useful in industrial applications where flocculation is of primary importance (e.g. for sedimenting yeast during the brewing process). Thus according to a fourth aspect of the invention there is provided a method of fermentation comprising growing yeast cells containing the *SRB1/PSA1* gene or functional

derivatives thereof operatively linked to a heterologous promoter in a growth medium in which *SRB1/PSA1* expression is regulated by the heterologous promoter whereby said cells flocculate.

*SRB1/PSA1* expression may be regulated in cells used according to the fourth aspect of the invention by an ~~inducible~~ inducible promoter or a constitutive promoter. *pMET3* is a preferred promoter for regulating *SRB1/PSA1* expression in cells used according to the fourth aspect of the invention.

Examples of cells in which *SRB1/PSA1* is under the regulation of an ~~inducible~~ inducible promoter include:

- (i) ZO125 (ZO 123 cells transformed with *pMET3-SRB1*); and
- (ii) FY23*SRB1MET3*.

Although we do not wish to be bound by any hypothesis we believe that the flocculation phenotype caused when *SRB1/PSA1* is transcribed from *pMET3* is not due to the gene's underexpression but, rather, is the result of its constitutive expression. Cell viability is not affected by the constitutive expression of *SRB1/PSA1*, suggesting that sufficient *Srb1/Psa1p* is synthesised under the control of *pMET3* to allow yeast to go through its cell cycle. However, when *SRB1/PSA1* is expressed from its own promoter, its transcription level increases some 4- to 6-fold at START. Thus the constitutive expression of *SRB1/PSA1* from *pMET3* could hyperactivate glycosylation at all other cell cycle phase which may lead to enhanced cell growth and flocculation.

According to a fifth aspect of the invention there is provided a method of fermentation comprising growing yeast cells containing the *SRB1/PSA1* and *PKC1* gene or functional derivatives thereof operatively linked to a heterologous promoter in a growth medium in which

*SRB1/PSA1* and *PKC1* expression is regulated by the heterologous promoter whereby said cells flocculate.

We believe cells used according to the method of the fifth aspect of the invention have a flocculating phenotype because *SRB1/PSA1* is not regulated by its endogenous promoter in such cells. These cells may comprise:

(i) *PKC1* operatively linked to an ~~inducible~~ inducible promoter and *SRB1/PSA1* linked to any heterologous promoter; or

(ii) both *PKC1* and *SRB1/PSA1* operatively linked to an ~~inducible~~ inducible promoter (i.e. cells according to the first aspect of the invention).

The method of the fifth aspect of the invention may be used when it is desirable to induce lysis (e.g. according to the method of the second aspect of the invention) at a predetermined time during the fermentation as well as flocculation. For instance, this may be achieved by adding methionine (0.05mM - 20 mM) to the growth medium when *PKC1* is operatively linked to a methionine regulated promoter such as *pMET3*.

According to a sixth aspect of the invention, there is provided a yeast cell containing the *PKC1* gene or functional derivatives thereof operatively linked to a heterologous ~~inducible~~ inducible promoter.

Cells according to the sixth aspect of the invention may be employed in the method according to the third aspect of the invention. Such cells may contain the *PKC1* gene or functional derivatives thereof operatively linked to any ~~inducible~~ inducible promoter described above for use in cells according to the first aspect of the invention.

Preferred cells according to the sixth aspect of the invention include:

- (i) ZO124 transformed with pRS316-p*MET3-PKC1*, pRS316-F<sub>1</sub>F<sub>2</sub>-p*MET3-PKC1* or pRS316-F<sub>1</sub>F<sub>2</sub>-TRP1-p*MET3-PKC1* (see Example 1);
- (ii) ZO123 transformed with pRS316-p*MET3-PKC1* or p*MET3-PKC1* containing fragments derived from pRS316-F<sub>1</sub> F<sub>2</sub>-p*MET3-PKC1* or pRS316-F<sub>1</sub>F<sub>2</sub>-TRP1-p*MET3-PKC1* (see Example 1); and
- (iii) yeast strain ZO-126 (see Example 2).

According to a seventh aspect of the invention, there is provided a yeast cell containing the *SRB1/PSA1* gene or functional derivatives thereof operatively linked to a heterologous promoter.

Cells according to the seventh aspect of the invention may be employed in the method according to the fourth aspect of the invention. Such cells may contain the *SRB1/PSA1* gene or functional derivatives thereof operatively linked to any heterologous promoter (including ~~indueable~~ inducible promoters). Preferred promoters are described above for use in cells according to the first aspect of the invention.

Examples of cells according to the seventh aspect of the invention include:

- (i) ZO125 (ZO 123 cells transformed with p*MET3-SRB1*); and
- (ii) FY23*SRB1MET3*.

According to a eighth aspect of the invention, there is provided a yeast cell containing the *PKC1* gene or a functional derivative thereof operatively linked to a heterologous ~~indueable~~ inducible promoter and the *SRB1/PSA1* gene or a functional derivative thereof operatively linked to a heterologous promoter.

Please replace the paragraph beginning at page 20, line 2, with the following amended paragraph:

Yeast according to the first aspect of the invention were made by transforming suitable yeast strains and the extent of ~~inducible~~ inducible cell lysis assessed relative to single *SRB1* and *PKC1* mutants.

Please replace the paragraph beginning at page 30, line 24, with the following amended paragraph:

Cells of strain ZO127, in which integrated copies of both *SRB1/PSA1* and *PKC1* are expressed from the *MET3* promoter, were grown in minimal medium until early exponential phase, methionine was then added to repress this expression. The results (Fig. 4) show that, upon addition of methionine, cells lost viability more quickly than those having either of the single *pMET3* expression cassettes (compare these results with those in Figs 2 and 3). Within 24 h, more than 80% of the cells lysed, releasing the bulk of their intracellular protein into the growth medium. These results indicate that the lysis phenotype conferred by the repression of *SRB1/PSA1* and *PKC1* is additive. Cells in which both *SRB1/PSA1* and *PKC1* expression may be regulated are particularly suited for use in the biotechnology industry in applications where ~~inducible~~ inducible lysis is required

Please replace the paragraph beginning at page 37, line 23, with the following amended paragraph:

Flocculation provides a means of efficient separation of cells from the medium. However, most cell wall mutants with enhanced flocculation capacity are affected in cell growth or cell viability, like *KRE6* and *mn9*. The flocculation resulting from *SRB1/PSA1* expression from the *MET3* promoter has no overt effect on cell growth and viability. Thus cells in which *SRB1* is under the regulation of an ~~inducible~~ inducible promoter may be used industrially when flocculation only is required.